Fish Physiology and Biochemistry (2005) 31: 33–44
DOI 10.1007/s10695-005-4587-1

Starvation induced alterations in hepatic lysine metabolism in different families of rainbow trout (*Oncorhynchus mykiss*)

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Received 29 July 2005; Accepted 31 October 2005

Key words: feed efficiency, lysine, lysine α-ketoglutarate reductase, rainbow trout

Abstract

Lysine is the second limiting amino acid in fish meal based diets, second only to methionine. However, little is known about lysine metabolism in rainbow trout (RBT). Therefore, lysine catabolism by the lysine α-ketoglutarate reductase (LKR) pathway was studied. Additionally, since genetically improved strains could influence fish production, these studies were performed in 4 distinct families of RBT. Two full-sibling families, differing in feed efficiency, were selected from each of 2 strains (A and B) of RBT. Eight fish from each of the 4 families were allotted to individual tanks. Fish were fed until satiation for 5 weeks when four fish within each family were randomly selected for 2 weeks of starvation. After the starvation period, all fish were harvested. Hepatic *in-vitro* LKR activity and lysine oxidation were measured as was LKR mRNA. No effect of family within strain on LKR activity or lysine oxidation was detected. Strain A exhibited a 55% reduction ($p < 0.01$) in LKR transcripts compared to strain B pooled across both feeding levels. Within each family, LKR mRNA was decreased ($p < 0.01$) in starved vs. fed fish. On average, there was a 68% decrease in LKR transcripts for starved fish. LKR activity averaged 104 ± 33 and 150 ± 31 nmol/min*gm liver ($p > 0.1$) in fed and starved fish, respectively. Lysine oxidation averaged 1.2 ± 0.5 and 2.2 ± 0.4 nmol/min*gm liver ($p > 0.1$) in fed and starved fish, respectively. LKR transcripts were positively correlated to weight gain ($p < 0.01$). These data are consistent with multiple modes of LKR regulation in fish.

Abbreviations: Feed Conversion Ratio – FCR; Lysine α-*ketoglutarate reductase* – LKR; Lysine Oxidation – LOX; Rainbow Trout – RBT; Saccharopine Dehydrogenase – SACDH.

Introduction

Aquaculture is rapidly growing in the United States. The USDA estimates that over a billion dollars worth of aquaculture products were sold in 2002 with approximately 40 million pounds of rainbow trout (RBT), *Oncorhynchus mykiss*, contributing to these sales. As aquaculture expands, research has been aimed at not only reducing production costs but also reducing the environmental impact of aquatic agriculture. For carnivorous fish, like RBT, acquiring more knowledge about amino acid metabolism will assist in the development of more environmentally sustainable and cost efficient production strategies. The essential amino acid lysine is the second most limiting amino acid in fish meal based diets, second only to methionine. However, little is known about

lysine metabolism in RBT. Improving lysine utilization in RBT would benefit the aquaculture industry in several ways.

First, carnivorous fish, like RBT, are metabolically adept at using protein as an energy source (Cho and Kauchik 1990; Kim et al. 1991) and require a much higher concentration of dietary protein ($>40\%$) relative to other animals in agricultural systems (6–20%). Higher protein diets are, on average, more expensive. Therefore, in aquatic animal culture, feed costs can account for 30–70% of production costs (Shang and Tomasso 1990).

Second, nitrogenous waste products are primarily of dietary origin (Gatlin and Hardy 2002). Many studies in fish have demonstrated significant reductions in total ammonia nitrogen excretion by reducing dietary crude protein and supplementing with lysine (Viola and Lahav 1991; Viola et al. 1992; Rodehutscord et al. 1994). Recently, Cheng et al. (2003b) reported a significant decrease of total ammonia excretion and soluble-phosphorus in effluent from RBT fed plant based diets supplemented with 1.9–2.25% lysine over diets supplemented with 1.5–1.8% lysine.

Third, RBT feeds usually contain fish meal as the primary protein source (Hardy 1999). However, fish meal production is not increasing and may not meet future demands of producers (Cheng et al. 2003a). Thus, plant protein is increasingly used to replace fish meal as a protein source in fish feed. For RBT diets, plant protein meal is often low in lysine relative to fish meal, therefore lysine supplementation may be necessary for optimal growth (Cheng et al. 2003a). Lysine supplementation of plant based diets has been shown to significantly improve growth performance of RBT (Cheng et al. 2003a). Understanding lysine degradation in RBT will provide insight for improving lysine utilization thus potentially ameliorating the need for lysine supplementation to plant based diets.

Much less is known about lysine oxidation in fish compared to other agricultural species. Lysine catabolism by the lysine α-ketoglutarate reductase (LKR) pathway is the presumed major route of lysine degradation in fish, since the LKR pathway is believed to be the predominant pathway for lysine degradation in mammals (Broquist 1991). The LKR pathway consists of several enzymatic steps. First, LKR reduces lysine in a NADPH-dependent step to saccharopine. The saccharopine is then oxidized in a NAD^+-dependent step to produce α-aminoadipate-γ-semialdehyde and glutamate by saccharopine dehydrogenase (SACDH – Figure 1). According to cDNA sequence analysis, the LKR and SACDH activities are on one bifunctional enzyme in RBT (GenBank accession no. AY751465), similar to both plants and mammals (Epelbaum et al. 1997; Papes et al. 1999).

Research on the regulation of LKR activity has mostly been focused in mammals and plants. Papes et al. (1999) reported an increase in LKR mRNA abundance and LKR activity in mice upon injections of lysine. In the same study, starved mice also showed an increase in LKR mRNA and LKR activity relative to fed controls. Interestingly, in plants, phosphorylation-dependent activity has been observed in purified LKR protein from soybeans (Miron et al. 1997). These data support both transcriptional and post-translational mechanisms for regulating enzyme activity. Thus, multiple

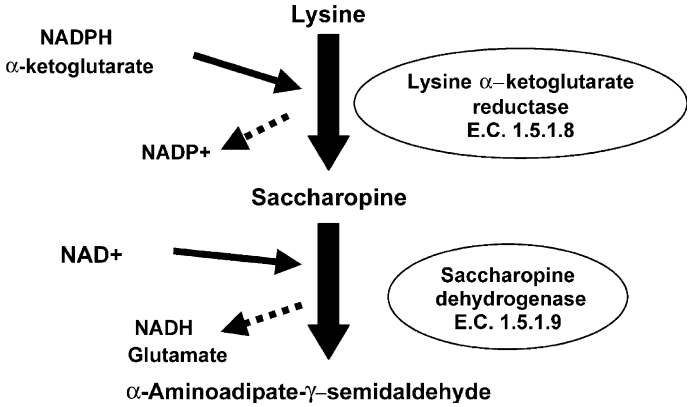

Figure 1. First enzymatic reaction in LKR pathway.

modes of regulation for LKR activity may exist in fish, as well.

In mammals, the activities of many different amino acid degrading enzymes increase as protein intake increases (Harper 1965), which is similar in fish (Kim et al. 1992). Unfortunately, there are no reports on the effect of dietary protein level on LKR activity if fish. However, Walton et al. (1984) reported no significant increase in hepatic LKR activity for RBT fed increasing amounts of dietary lysine, which contradicts what has been reported for LKR in rats and chickens (Wang et al. 1973; Chu and Hegsted 1976). Therefore, the regulation of LKR activity and lysine degradation may be different in fish compared to other species.

Understanding the basic mechanisms of lysine degradation and specifically LKR activity may enable the development of genetic tools to manipulate lysine degradation which could have significant value to the aquaculture industry. Hence, this study investigated the regulation of LKR activity and lysine oxidation (LOX) in RBT by measuring the effects of fasting on hepatic LKR activity, LKR mRNA abundance and LOX. This study is the first in fish to examine LKR gene expression as affected by nutritional status. Moreover, these studies were performed in 4 genetic groups (full-sibling families) of RBT previously identified based on differences in feed conversion ratios to determine if there were relationships between lysine metabolism and fish performance.

Materials and methods

Reagents and chemicals

Commercial fish feed was purchased from Ziegler Brothers, Inc. (Gardners, PA). Triton X-100, L-lysine monohydrochloride and 2-mercaptoethanol were purchased from Fisher Scientific (Fair Lawn, New Jersey). Biosafe-II scintillation fluid was from Research Products Inc. (Mount Prospect, IL). SYBR Green was obtained from Molecular Probes (Eugene, Oregon). IQ Supermix and flourescein were from Biorad (Hercules, CA). Random primers, RQ1 RNase free DNase and M-MLV Reverse Transcriptase were from Promega Corp. (Madison, WI). Trizol, 125:24:1 mixture of acid–phenol: chloroform: isoamyl alcohol and 25:24:1 mixture of phenol: chloroform: isoamyl alcohol were from Ambion (Austin, TX). Gene specific primers were synthesized by Invitrogen (Carlsbad, CA). All other reagents were from Sigma-Aldrich (St. Louis, MO).

Fish

Two previously described (Silverstein et al. 2005) strains of RBT with a long history of domestication were used in this study. Strain A originated from the alpine lakes in Washington State. Strain B was developed from a cross between a Puget Sound Steelhead and a Canadian Kamloops strain. As part of a preliminary study to examine genetic differences in growth and feed efficiency, 11 full-sibling families of RBT were generated at the USDA-ARS National Center for Cool and Cold Water Aquaculture (NCCCWA); 5 full-sibling families were from strain A and 6 full-sibling families were from Strain B. The highest and lowest feed efficiency full-sibling families were identified within both strains for a total of 4 families. The efficiency classifications were based on a 5-week feeding trial using 5 individually housed fish from each family and feeding them to apparent satiation (until pellets were left uneaten for 1 min) twice daily. The amount of feed consumed was measured by weighing the feed containers before and after feeding. The fish weighed approximately 2–4 g and grew to approximately 10 g during the preliminary study. The remaining fish from these families were maintained in 200 l full-sibling family tanks until the start of the study.

From each of the 4 families identified in the preliminary study, 8 fish of similar weight were obtained. At the start of the study, in August, the weight of the fish was 72 ± 2 g. All 32 fish were randomly allotted into 9 l individual tanks. The rearing system was a recirculating system using a biofilter, where 15% of the water was exchanged daily. The dissolved oxygen content was near saturation. Water temperature ranged from 14.3 to 14.8 °C and a 12 h light cycle was used.

Experimental design (Figure 2)

Fish were fed 2% of their body weight for a 2-week acclimation period during which time they resume normal feed consumption. All fish were fed a commercial diet (42% crude protein). However,

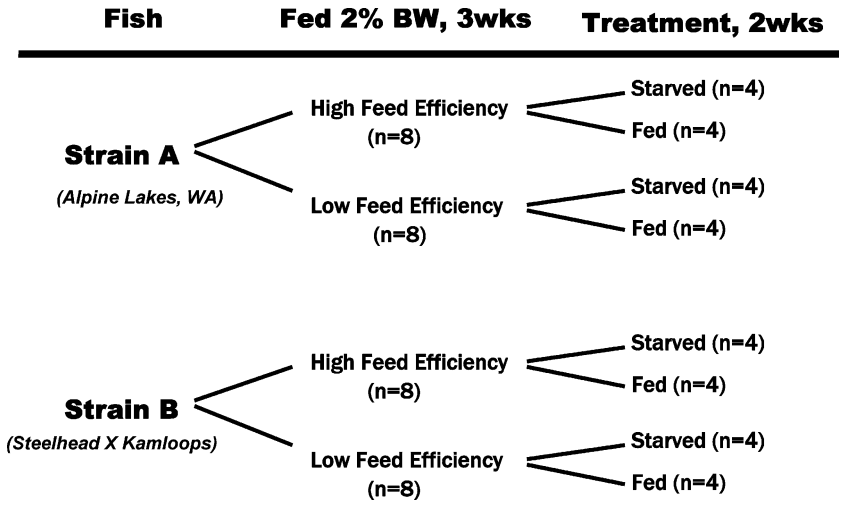

Figure 2. Experimental Design. All 32 fish were randomly distributed into individual tanks with a water temperature between 14.3 and 14.8 °C and subject to a 12 h light cycle. Fed fish received their final meal 24 h prior to harvest.

the data on two fish, which did not start eating but were not replaced, is excluded from the statistical analysis. After the acclimation period, all fish continued to be fed 2% of their body weight for the next 3 weeks. At the end of 3 weeks, 4 fish from each family were randomly selected to be starved for 2 weeks. At the end of the starvation period, all 32 fish were harvested. The fed fish received their final meal 24 h prior to harvest. Body and liver weight were recorded for each fish. Approximately 0.2 g of liver from the upper lobe was snap frozen in liquid nitrogen and the remaining liver placed in ice cold H buffer (5 mM HEPES, 5 mM 2-mercaptoethanol, 1 mM EGTA, 220 mM mannitol, 70 mM sucrose, 0.05% (w/v) bovine serum albumin, pH = 7.4). The livers in H buffer were transported on ice from Leetown, WV to Morgantown, WV. Snap frozen tissue was kept at −80 °C until RNA was extracted. The LKR activity and lysine oxidation assays were performed the day the fish were harvested.

Lysine oxidation assay

In-vitro hepatic lysine oxidation was assessed by measuring the recovery of $^{14}CO_2$ from [U−^{14}C] L-lysine in a procedure described by Blemings et al. (1998). Briefly, using a polytron, each liver was homogenized in enough H Buffer to make a 25% (w/v) homogenate. Incubations (final volume 2 ml) were started when homogenate (1 ml) was added to a 25 ml Erlenmeyer flask containing the following reagents (final concentrations): 10 mM L-lysine–HCl, 10 mM HEPES, 3 mM $MgCl_2$, 0.2 mM EDTA, 182 mM mannitol and 61 mM sucrose. The specific activity of [U−^{14}C] L-lysine was 4.2 Bq/nmol. Reactions were incubated for 30 min, while shaking (100 oscillations/min) in a water bath (25–27 °C). The incubation of liver homogenate with [U−^{14}C] L-lysine was shown to produce $^{14}CO_2$ in a linear fashion for at least 1 h. Carbon dioxide was trapped in a 1.5 ml conical centrifuge tube containing 0.5 ml of base trap solution (ethanolamine and methylcellosolve, 1:2). To terminate the reactions, 0.5 ml of 35% perchloric acid was injected through serum caps covering the flasks. To ensure maximal recovery of $^{14}CO_2$, flasks remained in the water bath with continuous shaking for an additional 180 min after incubations were acid-killed. The 1.5 ml conical centrifuge tubes were removed, placed in 17 ml of Biosafe-II scintillation fluid and vortexed. Radioactivity was determined in a Beckman LS 6500 (Beckman Coulter Inc, Somerset, NJ) liquid scintillation counter. The average of duplicate measures for each liver was used to determine total *in-vitro* hepatic lysine oxidation.

LKR assay

LKR activity was measured spectrophotometrically as the lysine-dependent oxidation of NADPH at room temperature. This procedure was first described by Hutzler and Dancis (1968),

performed on fish liver by Walton et al. (1984) and later modified in rat by Blemings et al. (1994). Enzyme activity was assayed when 25 μl of a 25% (w/v) liver homogenate and 25 μl of 10% Triton-X were added to a cuvette containing 850 μl of the following: 127.5 mM HEPES, 114.75 mM mannitol, 38.25 mM sucrose, 4.25 mM 2-mercaptoethanol, 0.0425% (w/v) bovine serum albumin, 0.21 mM NADPH, 12.75 mM α-keto glutarate and 0.05% (v/v) Triton-X 100. L-lysine solution or water was then added to bring the final volume to 1 ml, and the cuvette was covered with parafilm and gently inverted. The final concentration of lysine was 40 mM. The reported Michaelis Constant (K_m) of RBT LKR was 7.3 mM for lysine and 0.5 mM for α-ketoglutarate (Walton et al. 1984). Thus, concentrations of both lysine and α-ketoglutarate provided for near V_{max} conditions. A single assay consisted of 2 cuvettes with lysine and 2 cuvettes with water, which was conducted in duplicate for each liver homogenate. The assay was performed in a Beckmam Coulter DU 640 spectrophotometer and was linear for 3 min.

Real time PCR

Primers were designed to RBT-LKR based on RBT sequence (Genbank accession no. AY751465) provided by the NCCCWA. The forward and reverse LKR primer sequences 5′–3′ are GCG AGT GCT ACT ACT GGG TTC and CCT CTG CCT GGG TCA ACA AC, respectively. Acidic Ribosomal Protein P_o (ARP) was used for the housekeeping gene. ARP primers were previously published (Pierce et al. 2004). The size of the PCR products for LKR and ARP were 113 and 112 base pairs, respectively. PCR products were sequenced and primers were confirmed to be specific. PCR efficiencies (Pfaffl 2001) for ARP and LKR were 1.84 ± 0.02 and 1.95 ± 0.02, respectively. Melt curve analysis showed a single product for both LKR and ARP with no primer dimers even at low cDNA concentrations. The intra-assay and inter-assay coefficient of variation were $< 1.4\%$ for non-transformed C_t values (Pfaffl 2001).

Approximately 200 mg of frozen liver from each fish was used to isolate total RNA. RNA was extracted using TRIZOL (Invitrogen) following the manufacturer's protocol. However, prior to precipitation with isopropanol the RNA was washed twice with a 125:24:1 mixture of acid phenol:chloroform:isoamyl alcohol, and then washed twice again with a 25:24:1 mixture of phenol:chloroform:isoamyl alcohol. For determination of C_t values, 8 livers, one from each treatment within each family, were processed and assayed together at one time. RNA quality was assessed using OD_{260}/OD_{280} nm absorption ratio and a denaturing gel containing 2.2 M formaldhyde. Ten microliters from each RNA sample was pooled. This pool was used with each reverse transcription and real-time PCR reaction to serve as a reference for inter-assay variation due to differences in reverse transcription efficiencies. For each liver sample and the pool, 2 μg of total RNA was DNase treated with 2 units of RQ1 RNase-Free DNase and reverse transcribed using 1 μg random primers and 200 units of M-MLV reverse transcriptase.

Each cDNA sample was analyzed in separate reaction tubes for LKR and ARP in triplicate PCR reactions (30 s 95 °C $\rightarrow$ 30 s 57 °C $\rightarrow$ 30 s 72 °C for 40 cycles) on the same 96-well plate. The cDNA generated from the reference pool was included on the plate as template for both LKR and ARP primers. A total of 4 plates were needed for all 32 livers. Real time PCR reactions contained 12.5 μl of BioRad IQ Supermix, 0.5 μl Forward LKR primer (50 micromolar) or ARP Primer (25 μM), 0.5 μl Reverse LKR primer (50 μM) or ARP Primer (25 μM), 0.25 μl Fluorescein (1 μM), 2.5 μl SYBR Green ($1\times10{,}000$ dilution), 6.75 μl DEPC-treated water and 2 μl of cDNA. Data was analyzed using an ''efficiency corrected relative expression'' as shown by Equation 1. below (Pfaffl 2001).

$$\text{Ratio} = \frac{E_{\text{LKR}}^{C_t(\text{PoolLKR})}}{E_{\text{LKR}}^{C_t(\text{SampleLKR})}} \Bigg/ \frac{E_{\text{ARP}}^{C_t(\text{PoolARP})}}{E_{\text{ARP}}^{C_t(\text{SampleARP})}} \quad (1)$$

where E = PCR Efficiency, C_t = Threshold Cycle, LKR is lysine α-ketoglutarate reductase and ARP is acidic ribosomal protein. $E_{LKR} = 1.95$, $E_{ARP} = 1.84$.

Statistics

Statistical significance was assessed using analysis of variance with PC-SAS General Linear Models. A 3-factor randomized design was used. The main effects of strain, family within strain and starvation were tested. The effect of family within strain was tested since comparisons of all four families relative to each other was not a biologically important comparison. For statistical analysis, the mRNA relative abundance ratio was transformed, to comply with the assumptions of ANOVA, using the following equation: log (ratio – 0.25). Orthogonal contrasts were used to delineate the effect of starvation and family. Correlation was tested using Pearson correlation coefficients. Differences in the expression of the housekeeping gene (ARP) due to starvation were assessed by χ^2 analysis.

Results

For both strains, low feed efficiency families had numerically lower (improved) feed conversion ratios (FCR (feed/gain)) compared to the preliminary study (Table 1). Only the high feed efficiency family selected from strain B remained more efficient than the low feed efficiency family ($p < 0.06$). FCR did not correlate to final weight gains for fed fish. Weight gain was not significantly different between the two strains; however, for strain A only, the high feed efficiency family gained more weight than the low feed efficiency family (Table 1). The average weight gain of fed fish for strain A high and low feed efficiency families was 72.6 ± 5.1 and 41.5 ± 2.0 g, respectively ($p < 0.05$). For strain B, the families were not significantly different with respect to weight gain. With respect to final body weights, there was a significant effect of starvation as well as strain and a strain by treatment interaction was detected, but no effect of family within strain was detected (Table 2). Livers from starved fish weighed significantly less than fed fish (Table 2). The mean liver weight for starved fish was 0.75 ± 0.05 g compared to 2.48 ± 0.17 g for fed fish.

No significant differences in *in-vitro* lysine oxidation between strains or for families within strain were detected. No significant differences were detected for *in-vitro* hepatic lysine oxidation between fed and starved fish (Figure 3). The mean lysine oxidation per gram of liver for fed and starved fish was 1.2 ± 0.5 and 2.2 ± 0.4 nmol lysine oxidized/min* g liver, respectively (Figure 3a). However, when data were scaled to total liver weight, hepatic lysine oxidation for fed fish tended to be greater ($p = 0.12$) than for starved fish; 2.9 ± 0.7 compared to 1.5 ± 0.6 nmol lysine oxidized/min (Figure 3b).

No significant differences in LKR activity between strains or for families within strain were detected. LKR activity for starved and fed fish was not significantly different when expressed per gram of liver. The mean LKR activity for fed and starved fish per gram of liver was 104 ± 33 and

Table 1. Feed conversion ratios (FCR) and weight gain for fed fish[a]

Efficiency	FCR preliminary study	FCR	Weight gain (g) (Fed fish only)
Strain A			57.1 ± 7.4
Low	0.98 ± 0.08*	0.71 ± 0.03	41.5 ± 2.0**
High	0.81 ± 0.04	0.79 ± 0.18	72.6 ± 5.1
Strain B			74.6 ± 5.7
Low	1.13 ± 0.14**	0.89 ± 0.04*	70.5 ± 9.9
High	0.75 ± 0.15	0.78 ± 0.02	78.8 ± 6.4

[a]Feed conversion ratios (FCRs (feed/gain) were calculated as feed consumed per weight gained. The preliminary FCRs were calculated from fish weighing approximately 2 g and grown to approximately 10 g over a 5-week period. For the preliminary study, each FCR represents the mean of 5 individually housed fish. Subsequently, the FCRs were calculated from fish with a starting weight of approximately 70 g. Weight gain was calculated for individually housed fish fed for 5 weeks. Each FCR represents the average of 4 individually housed fish for Strain B and 3 individually house fish for strain A. Strain A and Strain B represents the mean of 6 and 8 fish, respectively. The weight gains in the rows for strain A and B represent the weight gains averaged over the two families within each strain. Within each column: *$p < 0.10$; **$p < 0.05$ applies to comparisons made between families within a strain for each study.

Table 2. Fish and liver weights[a]

Strain	Treatment	N	Final weight (Grams)	Liver weight (Grams)
A	Fed	6	125.0 ± 9.1	2.59 ± 0.30
	Starved	8	92.1 ± 4.3	0.81 ± 0.08
B	Fed	8	153.5 ± 8.3	2.39 ± 0.20
	Starved	8	92.9 ± 4.4	0.69 ± 0.04

[a]N (# of individual fish), Final Weight (final weight after 5 weeks) and Liver Weight (total liver weight). Each value is the mean ± SEM. Data are pooled across families within strain since no main effect of family within strain nor interaction of family within strain by treatment was detected for final weight.

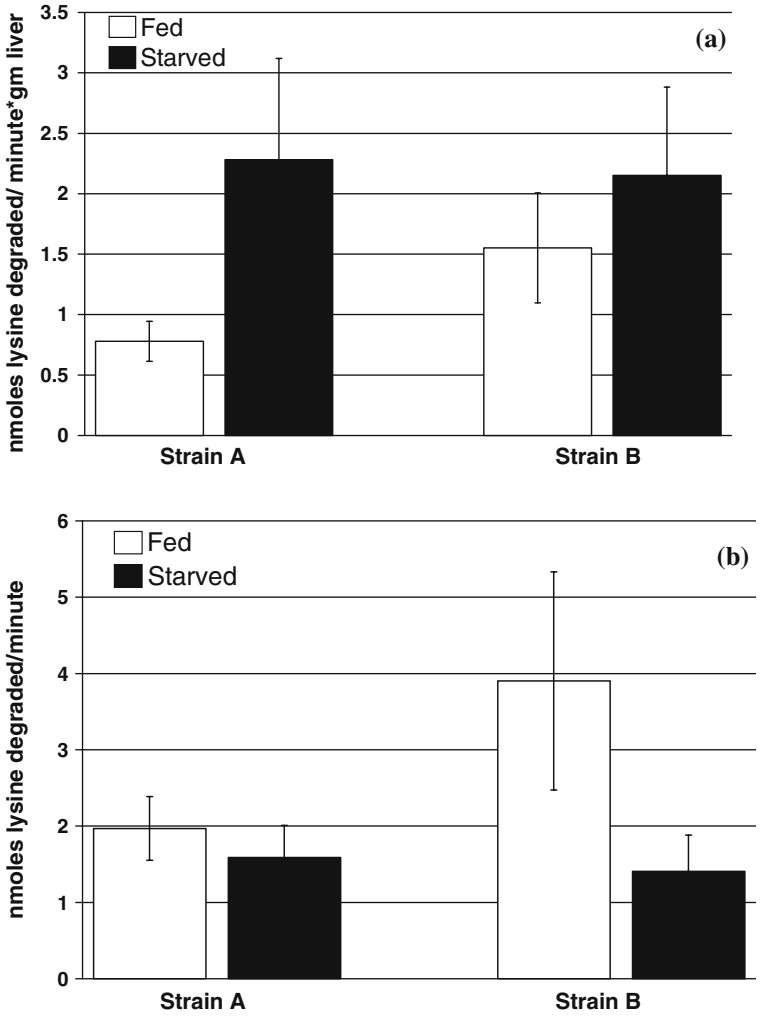

Figure 3. Lysine Oxidation (a) Lysine Oxidation per gram of liver (nmole lysine degraded per minute per gram of liver) (b) Total Hepatic Lysine Oxidation (nmole lysine degrade per minute). Each bar represents the mean of 8 fish ± SEM except for strain A fed, where the bar represents the mean of 6 fish.

150 ± 31 nmol lysine degraded/min*gm liver, respectively (Figure 4a). However, when data were scaled to total liver weight, LKR activity was less ($p < 0.05$) in the starved fish. Total hepatic LKR activity for fed fish is 272 ± 58 compared to 103 ± 53 nmol lysine degraded/min for starved fish (Figure 4b).

Interestingly, Strain B, pooled across both fed and starved fish, had significantly more LKR mRNA than Strain A ($p < 0.01$, Figure 5). However, relative LKR mRNA abundance was not significantly different for high and low feed efficiency families within either strain. For both strains, fish starved for 2 weeks had less ($p < 0.05$) LKR mRNA relative to ARP mRNA compared to fed fish (Figure 5). Starvation did not significantly affect ARP mRNA expression ($p > 0.10$). LKR mRNA abundance was not correlated to LKR activity or lysine oxidation but was positively correlated to weight gain ($p < 0.01$, Figure 6).

Discussion

Identifying metabolic mechanisms that may be responsible for phenotypic variation is important for RBT strain development. Studies that include both physiological and genetic variables may help

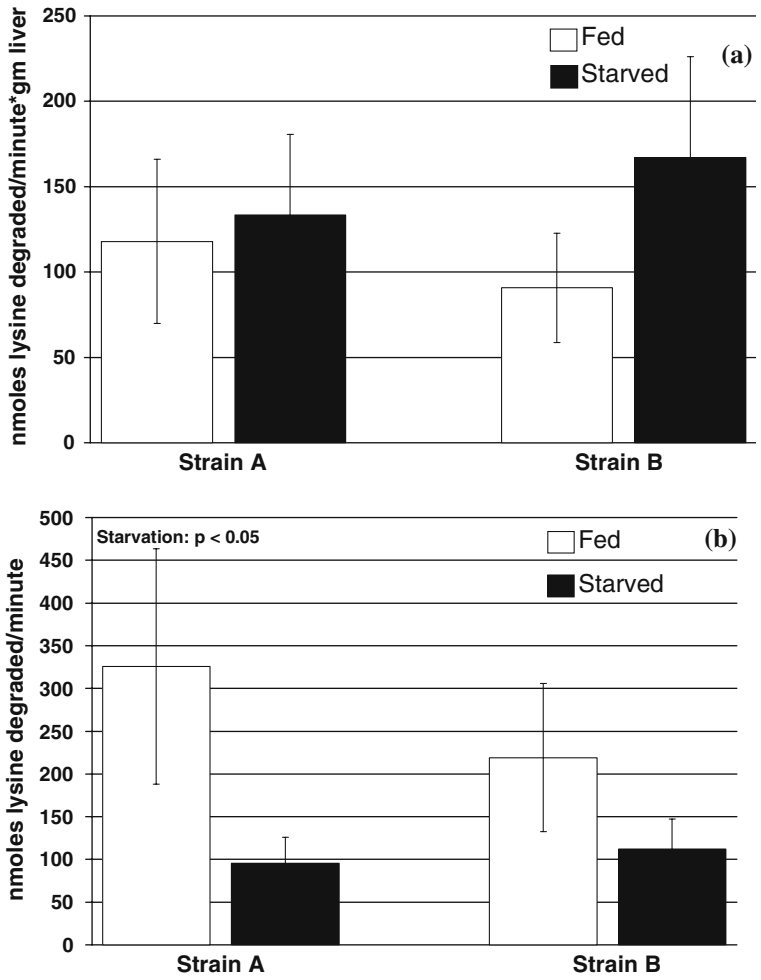

Figure 4. LKR Activity (a) LKR Activity per gram liver (nmole lysine degraded per minute per gram liver). (b) Total Hepatic LKR Activity (nmole lysine degrade per minute). Each bar represents the mean of 8 fish ± SEM except for strain A fed, where the bar represents the mean of 6 fish.

to elucidate how complex traits, like feed efficiency, are regulated in RBT (Silverstein 2004). The data presented here are the first to show LKR gene expression as affected by genotype and nutritional status.

The effects of selecting families with divergent feed efficiencies were observed. For the low efficiency families, the FCR changed as the fish aged. For both strains, the low efficiency families appeared as if they became more efficient with age, while high efficiency families' FCR changed very little (Table 1). These differences could have been due to the differences in feeding protocols between the two studies. Fish were fed to apparent satiation twice daily in the preliminary study but fed 2% of body weight in the subsequent study. It was important for the fish to maintain a similar plane of nutrition by receiving a fixed ration, since the goal was to assess differences in metabolism across strains and families. Therefore, differences in enzyme activity or gene transcription would reflect genetic differences not a difference in feed consumption.

The effect of genotype on lysine degradation is unclear based on these data. However, these data are the first and only measurements for LKR gene expression in RBT. Interestingly, the strains had significantly different amounts of LKR mRNA. Pooled across both fed and starved fish, strain B had 55% more LKR mRNA than strain A

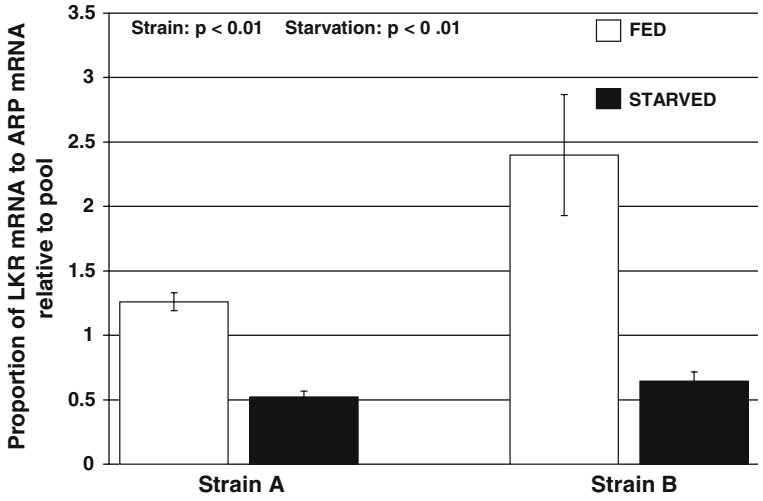

Figure 5. Proportion of LKR mRNA to ARP mRNA relative to pool. Each bar represents the mean of 8 fish ± SEM except for strain A fed, where the bar represents the mean of 6 fish. Main Effects: Strain ($p < 0.01$), Starvation ($p < 0.01$).

($p < 0.01$). However, this increase in mRNA for Strain B fish did not result in a significant increase in LKR activity relative to Strain A fish. Lysine oxidation, LKR activity (per gram of liver), and weight gain were not significantly different between the strains. The high and low feed efficiency families were not significantly different with respect to LKR mRNA relative abundance.

LKR is a mitochondrial enzyme in rat (Blemings et al. 1994) and has been suggested as such for RBT (Walton et al. 1984). Interestingly, the LKR activity for both the fed and starved fish is approximately 100-fold greater than the oxidation data, consistent with a mitochondrial location for LKR in RBT liver. Blemings et al. (1998) found that lysine oxidation in mitochondria is transport limited in rat liver thus, in trout liver total lysine oxidation may have been transport limited, as well. Moreover, using a subcellular targeting peptide prediction program (Emanuelsson et al. 2002), a mitochondrial targeting peptide was identified in the first 150 amino acids of RBT-LKR sequence.

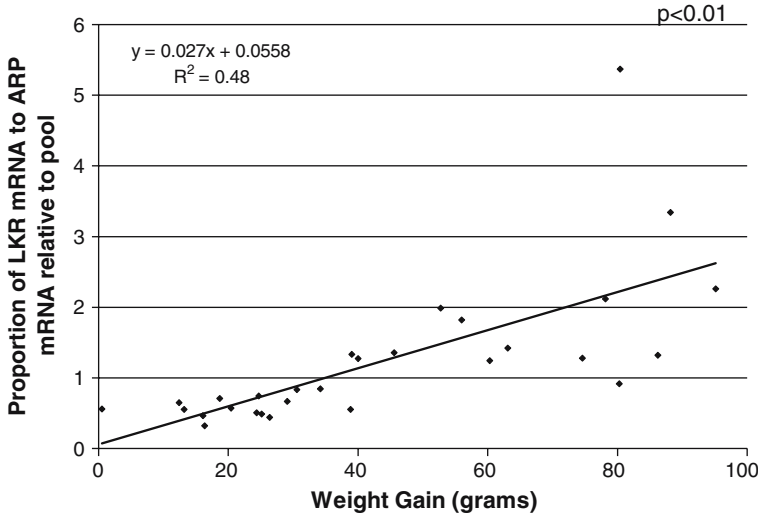

Figure 6. LKR mRNA abundance is positively correlated to weight gain Each dot represents a fish, including both starved and fed ($N = 29$).

LKR activity is, perhaps, modulated differently from other amino acid degrading enzymes. For example, histidase mRNA, *in-vitro* activity and protein levels have been found to increase in rats fed increasing amounts of protein in their diet, however, increasing the amount of histidine alone resulted in no increases (Torres et al. 1998). Contrary to histidase, LKR activity and LKR mRNA increase in mice receiving lysine injections or diets containing excess lysine (Foster et al. 1993; Papes et al. 1999). Moreover, Papes et al. (1999) observed an 80% increase in LKR mRNA and 52% increase in LKR activity in mice starved for 1 or 2 days relative to fed mice. The concomitant increase in LKR activity and LKR mRNA suggests enzyme activity is regulated via transcription. Here, we report a 68% decrease in LKR mRNA relative abundance for starved fish and a 50% increase in activity relative to fed fish. Moreover, LKR mRNA relative abundance is positively correlated with weight gain. Although these results seem paradoxical several explanations are possible.

First, the amount of mRNA does not necessarily relate to the amount of active LKR protein. The amount of LKR protein could have remained constant while a post-translational modification may have increased its activity. Phosphorylation-dependent activity of LKR has been reported in soybean (Miron et al. 1997). Moreover, amino acid analysis of RBT, mouse and human LKR revealed potential threonine and serine phosphorylation sites in both LKR and SACDH domains. Since antibodies for RBT-LKR are unavailable, it is not possible to quantify protein abundance at this time. However, in mice fed increasing amounts of casein (a milk protein), Kiess et al. (2004) reported a 100% increase in LKR activity but only a 10% increase in LKR mRNA abundance and 20% increase in LKR protein abundance. This suggests that there is an uncoupling of LKR activity, LKR mRNA and LKR protein abundance.

Second, during times of starvation, protein degradation would elevate blood lysine concentration, increasing liver lysine concentration, and, therefore, increasing flux thru the LKR pathway. The K_m of RBT-LKR is estimated to be 7.3 mM (Walton et al. 1984). This is well above the estimated lysine concentration in the blood of mammals at 0.2 mM and similar to the estimated concentration of 4 mM in liver mitochondria (Blemings et al. 1998). Therefore, LKR activity is probably substrate limited in RBT. Increasing LKR mRNA may not be necessary to increase the rate at which lysine is degraded since under *in vivo* conditions, substrate (lysine) concentration is well below the K_m. In contrast to RBT, mice starved for 1or 2 days exhibit a significant increase in LKR activity (Papes et al. 1999) despite an estimated K_m of 2.2 mM for rodents (Noda et al. 1978).

Third, the differences between these data and the results of Papes et al. (1999) may be a function of the relative state of starvation. The mice were starved for 1 or 2 days in contrast to a 2 week starvation period for the fish in this study. Certainly, homeothermic mice and poikilothermic fish are metabolically different. RBT can withstand several weeks of starvation whereas a mouse can not. The cellular response time to an environmental stress, such as starvation, may be different for fish compared to mammals. Fish enter into a period of hypometabolism when food is limited. Foster et al. (1991) reported an overall decrease in hepatocyte CO_2 production in perch starved for 7 weeks. The regulation of this metabolic flux between periods of hypometabolism and hypermetabolism may be exceptionally adapted for animals who survive long periods of limited food intake. The decrease in LKR mRNA abundance in starved fish relative to fed fish may reflect an overall decrease in metabolic enzymes as starved fish enter into a hypometabolic state.

A point is to be made regarding the positive correlation of LKR mRNA and weight gain. The fact that no correlation between LKR mRNA, LKR activity or LOX could be detected is attributed to the relatively high standard errors for these data. However, the lower variation in the LKR mRNA measurements may make it a more accurate estimate of LOX potential. In these diets, which were not lysine limiting, one would expect improved growth if more lysine could be oxidized consistent with Figure 6.

The response of metabolic pathways to stressors, such as starvation, may help to reveal how amino acid oxidation is regulated. Since carnivorous fish are adept at using protein as an energy source, amino acid oxidation may be less tightly regulated than in mammals. By examining the regulation of lysine degradation among different RBT strains, we are more likely to identify genotypic differences which may serve to decrease

the rate of lysine oxidation. In future studies, increasing the number of experimental units may be necessary to see significant differences in genetic lines of RBT. Moreover, evaluating LKR activity, mRNA and protein throughout the starvation period may help to elucidate the roles nutrients and genotype may have in regulating lysine oxidation in RBT.

Acknowledgements

We'd like to thank Dr. George Seidel for his statistical expertise. This work was supported by the WVU Research Corporation (KPB), the West Virginia Agriculture and Forestry Experiment Station (H413 – KPB), and the USDA (NRI 2002-35206-12859 & CSREES 2003-34386-13428-KPB). This is paper number 2939 of the West Virginia Agriculture and Forestry Experiment Station.

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